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RESEARCH PAPER

# The barley *amo*1 locus is tightly linked to the starch synthase *IIIa* gene and negatively regulates expression of granule-bound starch synthetic genes

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## Abstract

**In this study of barley starch synthesis, the interaction between mutations at the *sex*6 locus and the *amo*1 locus has been characterized. Four barley genotypes, the wild type, *sex*6, *amo*1, and the *amo*1*sex*6 double mutant, were generated by backcrossing the *sex*6 mutation present in Himalaya292 into the *amo*1 'high amylose Glacier'. The wild type, *amo*1, and *sex*6 genotypes gave starch phenotypes consistent with previous studies. However, the *amo*1*sex*6 double mutant yielded an unexpected phenotype, a significant increase in starch content relative to the *sex*6 phenotype. Amylose content (as a percentage of starch) was not increased above the level observed for the *sex*6 mutation alone; however, on a per seed basis, grain from lines containing the *amo*1 mutation (*amo*1 mutants and *amo*1*sex*6 double mutants) synthesize significantly more amylose than the wild-type lines and *sex*6 mutants. The level of granule-bound starch synthase I (GBSSI) protein in starch granules is increased in lines containing the *amo*1 mutation (*amo*1 and *amo*1*sex*6). In the *amo*1 genotype, starch synthase I (SSI), SSIIa, starch branching enzyme IIa (SBEIIa), and SBEIIb also markedly increased in the starch granules. Genetic mapping studies indicate that the *ssIIIa* gene is tightly linked to the *amo*1 locus, and the SSIIIa protein from the *amo*1 mutant has a leucine to arginine residue substitution in a conserved domain. Zymogram analysis indicates that the *amo*1 phenotype is not a consequence of total loss of enzymatic activity although it remains possible that the *amo*1 phenotype is underpinned by a more subtle change. It is therefore proposed that *amo*1 may be a negative regulator of other genes of starch synthesis.**

**Key words:** *amo*1, barley, GBSSI, mutant, *sex*6, SSIIIa, SSIIa, starch.

## Introduction

The endosperm of barley (*Hordeum vulgare* L.) grain typically contains ~50–60% starch composed of ~25% amylose and 75% amylopectin. Amylose is a mostly linear α-(1–4)-linked glucosyl chain with a few α-(1–6)-linked glucan chains and has a mol. wt of $10^4$–$10^5$ Da. Amylopectin is a highly branched glucan in which α-(1–4)-linked glucosyl chains (with 3– 60 glucosyl units) are connected by 4–5% α-(1,6) linkages, and has a mol. wt of $10^5$–$10^6$ Da. A suite of enzymes are involved in cereal starch biosynthesis, including ADP-glucose pyrophosphorylases (EC 2.7.7.27), starch synthases (EC 2.4.1.21), starch branching enzymes (SBEs; EC 2.4.1.18), and starch debranching enzymes (EC 3.2.1.41 and 3.2.1.68) (see reviews by Kossmann and Lloyd, 2000; Rahman *et al.*, 2000; Ball and Morell, 2003; James *et al.*, 2003; Tetlow *et al.*, 2004; Morell *et al.*, 2006).

Starch synthases transfer glucose from ADP-glucose to the non-reducing end of pre-existing α-(1–4)-linked glucosyl chains of starch. In higher plants, five classes of starch

synthases are consistently present; granule-bound starch synthase (GBSS), starch synthase I (SSI), SSII, SSIII, and SSIV (Li *et al.*, 2003). Ten starch synthase genes have been identified in the rice genome (Hirose and Terao, 2004); two GBSS isoforms (GBSSI and GBSSII), one SSI isoform, three SSII isoforms [SSIIa (also defined as SSII-3), SSIIb (SSII-2), and SSIIc (SSII-1)], two SSIII isoforms [SSIIIa (SSIII-2) and SSIIIb (SSIII-1)], and two SSIV isoforms [SSIVa (SSIV-1) and SSIVb (SSIV-2)] (Hirose and Terao, 2004; Fujita *et al.*, 2007). Proteins corresponding to SSI, SSIIa, and GBSSI have been detected within the starch granules from endosperm, whereas SSIIIa has only been detected in the soluble phase of amyloplastids (Li *et al.*, 2000).

Significant progress has been made in identifying roles of the starch synthases individually and cooperatively in determining the fine structure of starch. GBSS is essential for the biosynthesis of amylose (Nelson and Rines, 1962; Murata *et al.*, 1965; Eriksson, 1969; Delrue *et al.*, 1992; Nakamura *et al.*, 1995), and also contributes to the synthesis of the long chains of amylopectin (Maddelein *et al.*, 1994; Denyer *et al.*, 1996). GBSSI is present in endosperm, whereas GBSSII is present in leaves and stems.

Mutational analysis of the roles of SSI, SSIIa, and SSIIIa suggests that while each of these enzyme classes is primarily involved in amylopectin synthesis, each enzyme class is preferentially involved in the extension of specific subsets of chain lengths within the amylopectin molecule. Recent studies showed that SSI is involved in synthesis of the shorter outer chains of the amylopectin [degree of polymerizaton (DP)8–12] in leaf starch of *Arabidopsis* (Delvalle *et al.*, 2005) and in the endosperm starch of rice (Fujita *et al.*, 2006). Starch from barley and wheat SSIIa mutants showed an increase in chains of DP3–8, indicating that the SSIIa enzyme plays a role in extending shorter glucan chains of DP3–8 to longer glucan chains of DP12–35 (Yamamori *et al.*, 2000; Morell *et al.*, 2003; Konik-Rose *et al.*, 2007). Loss of SSIIIa in maize and rice confers an increased amylose phenotype, with a reduction in the proportion of very long chains in amylopectin (>DP50 in maize or >DP30 in rice), and slightly reduced gelatinization temperature (Jane *et al.*, 1999; Fujita *et al.*, 2007). *Arabidopsis* mutants defective for SSIV appear to have fewer, larger starch granules within the plastid, and a role in priming starch granule formation has been postulated for the SSIV protein (Roldan *et al.*, 2007).

The barley *amo*1 (high amylose Glacier, HAG) mutant was identified on the basis of an increased amylose phenotype, with an amylose content of up to 45% (Banks *et al.*, 1971). The *amo*1 mutation locus was mapped at 56.5 cM on chromosome 1H (by Andris Kleinhofs, Barley-BinMap 2005, GrainGene database). Although a number of groups have attempted to define the mutation locus, the nature of the *amo*1 locus remains to be defined (Boren *et al.*, 2008). The only gene involved in the starch synthesis pathway located on chromosome 1H is *ssIIIa* (Li *et al.*, 2000), and unpublished data (Z Li *et al.*) map this gene close to the *amo*1 locus, suggesting that *ssIIIa* is a candidate gene underpinning the *amo*1 mutation. The barley *sex*6 mutant is also a high amylose phenotype mutant, coupled with reduced starch content and reduced grain weight due to a reduction in starch biosynthesis (Morell *et al.*, 2003). This phenotype has been shown to result from the loss of the function of SSIIa enzyme in the endosperm (Morell *et al.*, 2003) which is encoded by the *ssIIa* gene on chromosome 7 of barley. The *sex*6 mutant barley produces starch with more short chains (DP6–11) and fewer intermediate chains (DP12–30) compared with the wild-type line. The high amylose content phenotype was due to the preferential reduction of the synthesis of amylopectin compared with that of amylose (Clarke *et al.*, 2008). Foods produced from the *sex*6 mutant barley give a low glycaemic index and contain high levels of resistant starch (Topping *et al.*, 2003).

In this study, the characterization of a barley population generated between the *sex*6 mutant (Himalaya292) and the *amo*1 mutant (HAG) in order to evaluate interactions between these recessive mutations in starch biosynthesis is described. The study resulted in the surprising observation that the combination of the recessive mutations results in a partial recovery of starch content and grain weight in the *amo*1*sex*6 double mutant, but without a statistically significant further increase in amylose content. The functions of the *amo*1 locus as a negative regulator of starch synthesis at elevated amylose levels are discussed.

## Materials and methods

### *Plant materials*

Barley lines used were from a population generated through three backcrosses between Himalaya292 (male, containing the *sex*6 mutation allele) (Morell *et al.*, 2003) and HAG (female, containing the *amo*1 mutation allele) (Banks *et al.*, 1971), followed by three generations of single seed descent. Grains from the third backcross were denoted as BC3F1 and from the third single seed descent were named as BC3F4. To increase the quantity of grains for each line, two further generations were grown (designated BC3F6 generations), which were used for this study.

In total, 71 BC3F6 barley lines were produced. These barley lines together with the parent and control barley lines, HAG, Glacier, Himalaya292, and Himalaya, were grown in the glasshouse at CSIRO Plant Industry, Canberra in pots in 2005 (with natural light and temperatures of 18 °C during the night and 24 °C during the day).

### *Cloning and sequencing of barley* ssIIIa *genomic DNA and cDNA*

For genomic DNA sequencing, genomic DNA from Himalaya, Himalaya292, Glacier, and HAG were used for the PCR amplification of fragments using three pairs of primers which were from the cDNA and genomic DNA sequence of the wheat *ssIIIa* gene (Li *et al.*, 2000; GenBank accession nos AF258608 and AF258609). Three pairs of primers were ZLSSIIIa-P1F (5′-ATGGAGATGTCTCTCTG GCCA-3′, located at nucleotide 29 of wheat *ssIIIa* cDNA), and ZLSSIIIa-P1R (5′-TCTGCATACCACCAATCGCCGT-3′, located at nucleotide 3806 of wheat *ssIIIa* genomic DNA); ZLSSIIIa-P2F (5′-ATCGTGACCTAACAGCTTTGGCG-3′, located at nucleotide 3189 of wheat *ssIIIa* genomic DNA) and ZLSSIIIa-P2R (5′-GAC AGAAGAACCCAAATCTGCGGTC-3′ located at nucleotide 7189 of wheat *ssIIIa* genomic DNA); and ZLSSIIIa-P3F (5′-GGAGGT CTCGGGGATGTTGTTAC-3′, located at nucleotide 6038 of wheat *ssIIIa* genomic DNA) and ZLSSIIIa-P3R (5′-CCACAAATGTAAA TATCATTGATGTAT-3′, located at nucleotide 9524 of wheat *ssIIIa* genomic DNA).

For cDNA sequencing, total RNA was extracted from the developing endosperm [15 days post-anthesis (DPA) was used due to the comparative high level of expression of starch synthase genes at this stage] of barley, Himalaya. The procedures for RNA extraction were as detailed in Clarke and Rahman (2005). First-strand cDNA was synthesized and used for PCR amplification of *ssIIIa* cDNAs. Primers used for amplification of the full-length of cDNA sequence were ZLSSIIIa-P1F and ZLSSIIIa-P4R (5′-ACGTCACTGCGGTTCTTATCTCG-3′, located at nucleotide 9403, after the stop codon of the genomic DNA sequence of wheat *ssIIIa*).

For each PCR (20 μl), 50 ng of cDNA or genomic DNA, 1.5 mM $MgCl_2$, 0.125 mM of each dNTP, 10 pmol of primers, 0.5 M glycine betaine, 1 μl of dimethylsulphoxide (DMSO), and 1.5 U of Advantage 2 Taq polymerase mix (Clontech) were used. The PCRs were conducted using a HYBAID PCR Express (Integrated Sciences) with one cycle of 95 °C for 5 min, 35 cycles of 94 °C for 45 s, 59 °C for 30 s, and 72 °C for 3 min, one cycle of 72 °C for 10 min, and one cycle of 25 °C for 1 min. The PCR fragments (1 μl) were cloned into a pCR2.1 TOPO cloning vector (Invitrogen). DNA sequencing was performed using the automated ABI system with dye terminators as described by the manufacturers at JCMRS, Australian National University, Australia.

DNA sequences were analysed using the GCG suite of programs (Devereaux *et al*., 1984) to detect single nucleotide polymorphisms (SNPs) in genomic DNAs of the *ssIIIa* gene from Himalaya292, Himalaya, Glacier, and HAG. A CAPS (cleaved amplified polymorphic sequence) marker was designed based on one SNP at nucleotide 6323 of barley *ssIIIa* genomic DNA between two parental lines which created an *Eco*RI site in the *ssIIIa* gene from HAG, but not from Himalaya292. Primer SSIIIa-P5F (5′-GGAGGTCTCGGGGATGT-3′) located at nucleotide 7442 bp and primer SSIIIa-P5R (5′-GGTTCCAGGAAGTAAACGGTCAGG-3′) located at nucleotide 7893 of the barley *ssIIIa* genomic DNA (GenBank accession numbers: Himalaya genomic DNA, JN256944; Himalaya292 genomic DNA, JN256945, Glacier genomic DNA, JN256946; HAG genomic DNA, JN256947) were used for the PCR amplification of the CAPS marker for the *ssIIIa* gene, generating a 464 bp product which was then digested with *Eco*RI.

### *Genotyping of the BC3F6 population by PCR amplification*

Young barley leaves from the BC3F6 population were collected and freeze-dried (freezer FTS systems, Stone Ridge, New York). Genomic DNA was isolated with a fast $DNA^R$ kit (Q-BIOgene, CA, USA).

For genotyping for the presence or absence of the *ssIIa* mutation from the *sex*6 mutant in Himalaya292, primer SSIIaF (5′-CCTGGAACACTTCAGACTGTACG-3′) starting at nucleotide 1616 and primer SSIIaR (5′-AGCATCACCAGCTGCACGTCCT-3′) starting at nucleotide 2044 of the *ssIIa* cDNA (GenBank accession no. AY133249) were used for the PCR amplification of a 451 bp product spanning the *ssIIa* mutation site (nucleotide 1829 of Himalaya292 as described; Morell *et al*., 2003).

The PCR products for the detection of the *ssIIa* mutation (for the *sex*6 locus) were digested with the restriction enzyme *Nla*IV at 37 °C overnight. Digested PCR fragments were separated on 2% agarose gels and visualized with gel documentary (UVitec) after GelRed (Biotium) staining. Three types of PCR fragment patterns were evident after electrophoresis on a 2% agarose gel that differentiated the mutated and wild-type *ssIIa* gene. Two DNA fragments (347 bp and 104 bp) indicated the occurrence of the mutated *ssIIa* gene from Himalaya292, three DNA fragments (236, 111, and 104 bp) indicated the presence of the wild-type *ssIIa* gene from HAG, and both 347 bp and 236 bp fragments (in addition to 111 bp and 104 bp fragments) indicated heterozygous genotype lines. The 104 bp DNA fragment was not used as it is not specific to the mutation. The 111 bp fragment could not be separated from the 104 bp fragment.

The *amo*1 mutation locus was mapped at 56.5 cM on chromosome 1H (by Andris Kleinhofs, Barley-BinMap 2005, GrainGene database). To genotype for the presence or absence of the region containing the *amo*1 mutation, 12 SSR (simple sequence repeat) markers (Ramsay *et al*., 2000) located between 56.00 cM and 64.60 cM and one *Eco*RI CAPS marker for the *ssIIIa* gene (this work), on the short arm of chromosome 1H, were selected for the amplification of PCR products from the two parental lines, Himalaya292 and HAG. These SSR markers were EBmac0405, Bmag0105, Bmac0063, HVM20, Bmac0090, EBmac0560a, EBmac0501, Bmac0044, Bmac0032, Bmag0113, Bmag0211, and Bmag0350. The primers for these SSR markers were synthesized according to the sequences listed in the GrainGenes Database.

The PCRs were assembled as above except for a changes of primers. PCR for detection of the *ssIIa* mutation and *ssIIIa* gene was carried out as above using a HYBAID PCR Express (Integrated Sciences) except using GoTaq Hot Start Polymerase (Promega) instead of Advantage 2 Taq polymerase. The PCR conditions for the 12 SSR markers was one cycle of 95 °C for 4 min, 15 cycles of 94 °C for 30 s, from 65 °C to 50 °C with a 1 °C decrease in each cycle for 30 s, and 72 °C for 1 min 20 s, 30 cycles of 94 °C for 15 s, 50 °C for 15 s, and 72 °C for 45 s, and one cycle of 25 °C for 1 min.

The PCR products for the *ssIIIa* gene were digested with the restriction enzyme *Eco*RI at 37 °C overnight. Digested PCR fragments were separated on 2% agarose gels and visualized with gel documentary (UVitec) after GelRed (Biotium) staining. Two types of PCR fragment patterns were evident after 2% agarose gel electrophoresis that differentiated the *ssIIIa* gene from HAG or Himalaya292. A 464 bp DNA fragment only indicated the occurrence of the *ssIIIa* gene from Himalaya292, while both 303 bp and 161 bp DNA fragments indicated the presence of the *ssIIIa* gene from HAG. Three fragments (464, 303, and 161 bp) were detected in the heterozygous lines.

For the *amo*1 mutation, PCR products from the 12 SSR markers were separated on 2% agarose gels and also on an 3130×l Genetic Analyser (following the instructions of Applied Biosystems). Two out of the 12 SSR markers, EBmac0501 and Bmac0090, gave clearly different sized PCR fragments. On 2% agarose gels, the EBmac0501 marker gave three PCR fragmentation patterns for the BC3F6 population. A 167 bp fragment was detected from HAG, a 141 bp fragment was detected from Himalaya292, and both 167 bp and 141 bp (EBmac0501) fragments were detected in the heterozygous lines. Using the 3130×l Genetic Analyser, the Bmac0090 microsatellite marker gave three PCR fragmentation patterns for the BC3F6 population. A 234 bp fragment was detected from HAG, a 236 bp fragment was detected from Himalaya292, and both 236 bp and 234 bp fragments were detected in the heterozygous lines.

### *Analysis of the expression of mRNAs for* sslIIa *and* sslIIb *in the developing endosperm*

For real-time PCR analysis, total RNA was extracted from the developing endosperm (at 15 DPA) of barley from five lines of each of the four genotypes. The procedures for RNA extraction and the first-strand cDNA synthesis were as described above. Primers used for amplification of *ssIIIa* cDNA between nucleotides 3649 and 3814 (Himalaya cDNA, JN256948; Himalaya292 cDNA, JN256949, Glacier cDNA, JN256950; HAG cDNA, JN256951) were ZLSSIIIa-RTF (5′-TGGACAAGTCGAAAACCTGACCG-3′) and ZLSSIIIa-RTR (5′-GGCAATGTATTATATGTGGAGAAAGTCC-3′). Primers used for amplification of *ssIIIb* cDNA between nucleotides 434 and 590 (GenBank accession no. FN179378) were ZLSSIIIb-RTF (5′-GGAAAGGTTGAAGGCATCTCTG-3′) and ZLSSIIIb-RTR (5′-TGATATCTGGAGAAGAGCCACTC-3′). Primers for the amplification of a housekeeping gene, α-tubulin 2, cDNA between nucleotides 658 and 831 (GenBank accession no. Y08490) were ZLbTUB2F

(5′-AGTGTCCTGTCCACCCACTC-3′) and ZLbTUB (5′-CAAA CCTCAGGGAAGCAGTCA-3′).

For each PCR (20 µl), 100 ng of cDNA, 3.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol of primers, 1×SYBR Green I (Invitrogen), 1× PCR buffer (–$MgCl_2$), and 0.25 U of Platinum Taq DNA polymerase (Invitrogen) were used. The PCRs were conducted and analysed using a RotorGene 6000 (Corbett Life Science, Australia). The real-time PCR conditions were one cycle of 95 °C for 10 min, 45 cycles of 95 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s, and melting from 72 °C to 95 °C rising by 1 °C for each step. Comparative quantitation analysis was used to calculate the comparative expression. The comparative expression for each genotype was calculated by mean values of the comparative concentration for mRNAs for *ssIIIa* and *ssIIIb* individually divided by the mean value of the comparative concentration for mRNAs for α-tubulin 2.

### *Grain composition and starch analysis*

The analysis of the starch and grain composition of the BC3F6 population was conducted using all homozygous lines for both *ssIIa* and three markers for the *amo*1 locus lacking recombinations at the *amo*1 locus. These lines included all wild-type lines, lines containing the *amo*1 locus alone, lines containing the *sex*6 locus alone, and lines containing both the *amo*1 and *sex*6 mutations. For the analysis of water-soluble carbohydrates (WSCs), starch chain length distribution, and grain morphology a subset of four lines per genotype was used.

### *Starch content, starch extraction, and amylose content*

Barley grains were first ground to wholemeal using a Cyclone mill machine (Cyclote 1093, Tecator, Sweden). Starch content was assayed using the AACC method 76.13 with 20 mg of wholemeal for each of three replicate samples (Konik-Rose *et al*., 2007). Starch was isolated by a protease extraction method (Morrison *et al*., 1984) followed by washing with water and removal of the tailings. Starch was then freeze-dried. Amylose content was measured using a small-scale (2 mg of starch) iodine adsorption method (Morrison and Laignelet, 1983) with some modifications as described by Konik-Rose *et al*. (2007).

### *Starch chain length distribution*

Starch chain length distribution was measured by the method of O'Shea *et al*. (1998) using a P/ACE 5510 capillary electrophoresis system (Beckman Coulter, NSW Australia) with argon laser-induced fluorescence (LIF) detection.

### *Grain weight and plumpness*

Grain weights for each of the 71 BC3F6 lines were determined as the average grain weight of 100 grains with three replicates. Grain plumpness was genotyped as three categories: shrivelled, semi-plump, and plump.

### *Microscopic examination of barley grains and their cross-sections*

Transverse sections (~1 mm thick) of the middle part (the largest diameter section) of the barley grains were produced from four genotypes of lines (wild-type lines, *amo*1 mutants, *sex*6 mutants, and *amo*1*sex*6 double mutants) by cutting with razor blades. The sections and intact grains were then photographed with a Leica MZFLIII (Leica Microsystems, Germany) at ×12.5 amplification.

### *Protein and lipid contents*

Protein content was determined by measurement of nitrogen content using a mass spectrometry method employing a Europa 20-20 (electronics rebuilt by Sercon in 2006) isotope ratio mass spectrometer with an automated nitrogen and carbon analyser preparation system. From 3 mg to 8 mg of wholemeal of barley was used. A nitrogen to protein conversion factor of 6.25 was used for the calculation of the protein content in barley grains (Mosse, 1990). Lipid content was measured using the AOAC 983.23 method and AACC method 08-01 (AOA Chemists, 1990), respectively.

### *β-Glucan content and pentosan content*

β-Glucan was measured using the AACC method 32.23 using 20 mg of wholemeal for each of three replicate samples. Pentosan content was measured using the method from Bell (1985) employing 20 mg of wholemeal for each of three replicate samples.

### *Quantification of water-soluble carbohydrate contents*

WSCs were extracted from wholemeal following the method of Lunn and Hatch (1995) with some modifications. Barley wholemeal (100 mg) was extracted three times with 10 ml of 80% ethanol (v/v) in a boiling water bath for 10 min. The supernatants from each extraction were pooled, freeze-dried, and resuspended in 2 ml of milliQ water. The quantities of sucrose, glucose, fructose, maltose, and fructo-oligosaccharides were analysed by high-performance anion exchange chromatography (HPAEC) (Ruuska *et al*., 2006).

### *Analysis of starch granule-bound proteins and SSIIIa enzymatic activity*

Starch granule-bound proteins were isolated and separated by SDS–PAGE gel as described (Rahman *et al*., 1995). The proteins were then stained by silver staining (Li *et al*., 1999). The protein gels were scanned (Epson Perfection 2450 PHOTO; Epson America Inc., CA, USA).

Developing endosperms at 15 DPA were isolated and ground in a mortar and a pestle with 3 vols of extraction buffer [20 mM TRIS-HCl, pH 7.5, 5 mM dithiothreitol (DTT), and 1 mM pefabloc SC (Roche)] at 4 °C. The homogenate was then centrifuged at 10 000 *g* for 20 min at 4 °C and the supernatant (containing 20 µg of proteins) was used for analysis of SSIIIa activity by zymogram (Abel *et al*., 1996). A two-dimensional gel system was applied with an 8% acrylamide native gel as the first dimension gel and a zymogram gel (Abel *et al*., 1996) as the second dimension gel.

### *Statistical analyses of the relationship between genotypes and grain components or starch properties*

Statistical analyses were performed using Genstat version 9. Analysis of variance was performed for grain weight, starch content, amylose content, amylopectin content, protein content, lipid content, β-glucan content, pentosan content, and WSC content to obtain the least significant difference (LSD, $P < 0.05$), looking at variation between the genotypes.

## Results

A population of lines segregating for the presence or absence of mutations at the *sex*6 and *amo*1 loci was generated by performing three backcrosses from a *sex*6 mutant donor line (Himalaya292) into an *amo*1 mutant line (HAG). Three generations of single seed descent were performed from the BC3F2 lines in order to generate sufficient fixed genotypes to investigate the relative impact of the *sex*6 and *amo*1 mutation loci (alone and in combination) on starch synthesis, grain composition, and morphology.

### *Assignment of progeny lines to phenotypic and genotypic groupings*

Central to this study is the ability to assign the progeny lines accurately to genotypes. Because the genetic change underpinning the *sex*6 phenotype has previously been demonstrated to be a lesion in the *ssIIa* gene, the status of all lines at the *sex*6 locus could be unambiguously defined (Supplementary Table S1 available at *JXB* online). However, the *amo*1 mutation is defined by phenotype alone given that the causal gene has yet to be defined. Two lines of evidence (genotype grouping and phenotype grouping) were used to assign lines to the four possible genotype classes (wild type, *sex*6, *amo*1, and *amo*1*sex*6 double mutant).

*Genotypic grouping*: Given that the causal gene at the *amo*1 locus has not been identified, identification of the closest linked markers available was sought. SSR markers identified from barley mapping populations within ~10 cM of the *amo*1 locus were examined for polymorphism in this population, and two markers (EBmac0501 located at 58.0 cM, and Bmac0090 located at 58.0 cM) gave clear polymorphisms between the Himalaya292 (*sex*6) and HAG (*amo*1) parents. As the *ssIIIa* gene is located in this region of chromosome 1H (Z Li *et al*., unpublished data), an *ssIIIa* marker based on an *Eco*RI restriction polymorphism at nucleotide 6323 of barley *ssIIIa* between the Himalaya292 and HAG *ssIIIa* genes was also developed (Supplementary Table S2 and Fig. S1 at *JXB* online). Of the 71 lines from the backcross population, 13 lines had a wild-type phenotype at both the *sex*6 and *amo*1 loci, 13 carried the *sex*6 mutation alone, nine had the *amo*1 mutation alone (containing three DNA markers for the *amo*1 locus), and there were 13 lines with both the *sex*6 and *amo*1 mutations (Supplementary Table S1). In each of these lines, the three markers at the *amo*1 locus (EBmac0501, Bmac0090, and the *ssIIIa* marker) showed no recombination between with wild type and *amo*1 alleles. A further five lines were identified that were homozygous at the *sex*6 locus, and were homozygous for the three *amo*1 markers but contained recombination between these three markers (Supplementary Table S1). The remaining 18 lines were heterozygous at one of the four markers used and were excluded from the phenotypic analysis.

*Phenotypic grouping*: Figure 1 shows the relationship between amylose content and starch content for the 53 homozygous BC3F6 lines. For lines containing the wild-type *ssIIa* gene (triangular symbols) a clear separation into two phenotypic groupings could be made. The grouping with elevated amylose consistently contained the three markers from the *amo*1 locus (filled triangles) while the group with lower amylose content were wild type for all three *amo*1 markers (open triangles). The lines with the *sex*6 genotype (non-functional SSIIa lines) also revealed the presence of two phenotypic groupings, separated in this case not by amylose content but by starch content. The lower starch content group contained the *sex*6 allele and the wild-type *amo*1 locus (open diamonds), while the higher starch content group contained both the *sex*6 and *amo*1 loci (filled diamonds). Five lines with recombinations between the *amo*1 markers (three with wild-type alleles at *sex*6, filled triangles indicated by arrows; two with the mutant *sex*6 allele, filled diamonds indicated by arrows) were included in the phenotypic analysis. Alignment of the phenotypic and genotypic data for the five recombinant lines provided evidence demonstrating that of the three markers, the *ssIIIa* SNP marker is more tightly linked to the *amo*1 locus than either of the EBmac 0501 or Bmac0090 markers (see Fig. 1; Supplementary Table S1 at *JXB* online). Additional studies with larger populations are being used to examine whether further recombinants can be identified and to quantify the linkage between markers in the *amo*1 region and the trait (see later section in this study).

### *Grain physical parameters*

*Grain weight*: Grain weight data for the four genotype groups are given in Fig. 2A. There were no statistically significant differences between grain weights of the *amo*1 mutant lines and wild-type lines ($P < 0.05$). However, there were statistically significant differences ($P < 0.05$) between each of the *sex*6 mutants and *amo*1*sex*6 double mutant lines and each of the respective three remaining genotypes.

*Grain morphology*: Intact grains from four representative lines for the four genotypes were examined by stereoscopic microscopy on both the dorsal and crease sides (Fig. 3). Consistent with the grain weight data, the wild-type (Fig. 3A) and the *amo*1 mutant (Fig. 3B) lines produce plump well-filled grains, while *sex*6 mutant lines produce shrunken grains (Fig, 3C). The *amo*1*sex*6 double mutants (Fig,3D) yield grains with an intermediate phenotype, plumper than the shrunken grains of *sex*6 mutants yet not as well filled as the grains of the *amo*1 mutant and the wild-type line.

*Grain cross-section*: To illustrate the plumpness of the grains from these genotypes further, transverse sections at the widest mid section of the grain are shown in Fig. 3. The wild-type (Fig. 3E) and *amo*1 genotypes (Fig. 3F) yield fully filled endosperms, while the *sex*6 mutant line (Fig. 3G) produces incompletely filled grains with a considerable reduction in endosperm packing density. *Amo*1*sex*6 double mutant lines (Fig. 3H) showed an intermediate phenotype, with larger grain sections containing an endosperm that is more fully filled than the *sex*6 mutant and yet less well filled than that of the wild-type or *amo*1 mutant lines.

### *Starch content and composition*

*Starch content*: Starch content for the four genotypes is shown in Fig. 2B. Compared with the wild-type lines, *amo*1 mutants, *amo*1*sex*6 double mutants, and *sex*6 mutant lines contained 10.0, 23.7, and 45.1% less starch, respectively. These values were statistically different among the four genotypes ($P < 0.05$).

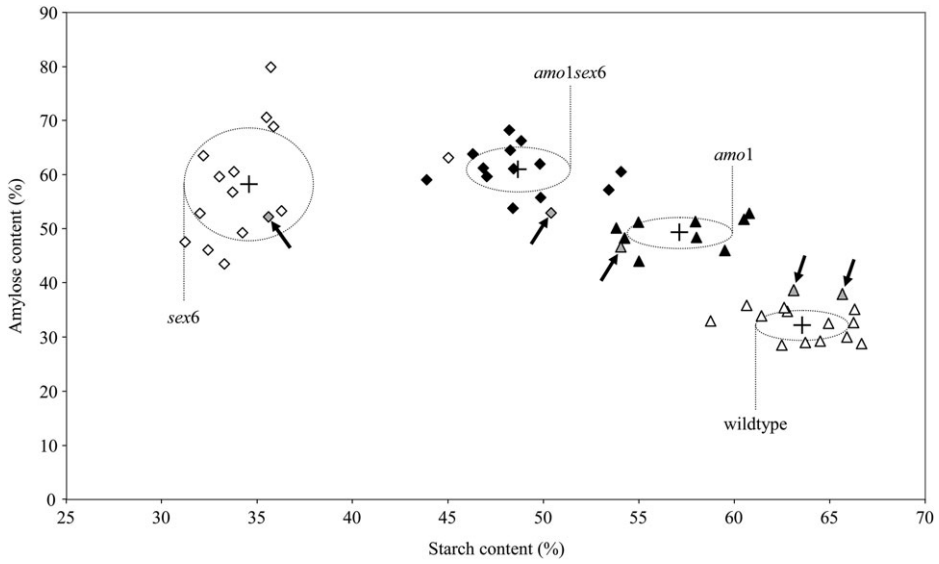


**Fig. 1.** Relationship between starch content and amylose content for homozygous genotypes from the Himalaya292×HAG BC3F6 lines grown in glasshouse conditions. Lines containing the wild-type allele at the *sex*6 locus are indicated by triangular symbols, whereas lines containing the inactivated *sex*6 allele are denoted by diamond-shaped symbols. Lines that contain homozygous wild-type markers at the *amo*1 locus are denoted by open symbols, lines with homozygous *amo*1 markers are denoted by filled symbols, and lines containing recombinations between the three markers at the *amo*1 locus have filled symbols indicated by arrows. For each of the four genotypes (wild type, open triangle; *amo*1, filled triangle; *sex*6, open diamond; *amo*1*sex*6, filled diamond) a cross indicates the coordinates of the average starch content and amylose content for that genotype and an ellipse marks the boundary of the region within one standard deviation of the mean for that genotype.

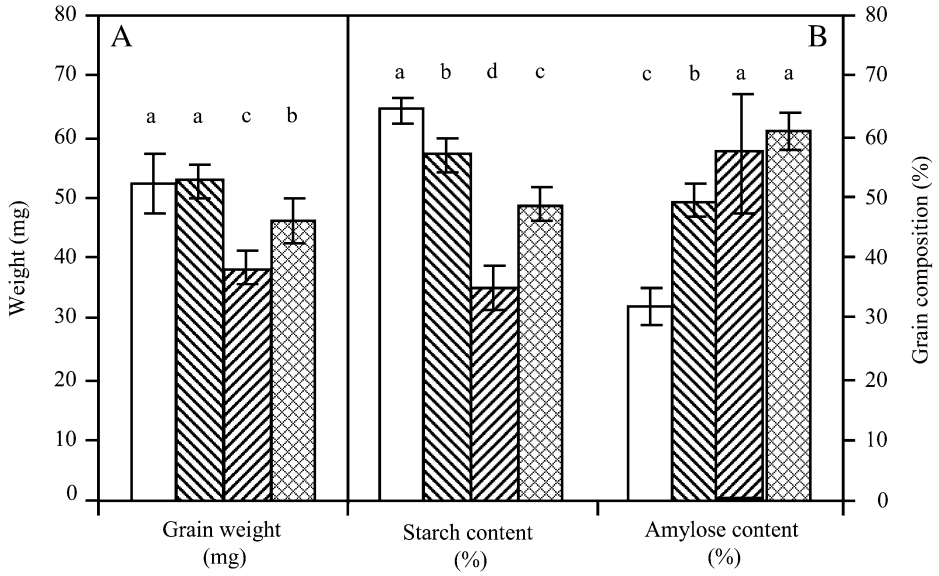


**Fig. 2.** Average grain weight (A), and starch content and amylose content (B) of the four genotypes of barley lines from the BC3F6 backcrossing population Himalaya292×HAG. The values on the *y*-axis represent mg for grain weight (left-hand side), or percentage (%) for starch content, and amylose contents (right-hand side). Starch content (%) indicates the percentage of grain weight accounted for by the starch. Amylose content (%) indicates the percentage of the starch isolated from barley grains accounted for by amylose. The standard error bars are labelled on the columns. The letters (a, b, c, and d) above the columns are based on the LSD; mean values with the same letter are not statistically significantly different, and those with different letters are statistically significantly different at $P$ <0.05. The four genotypes were the wild type (open box), *amo*1 mutant (box with downward slanting diagonal lines), *sex*6 mutant (box with upward slanting diagonal lines), and *amo*1*sex*6 double mutant (cross-hatched box).

*Amylose content*: Consistent with the data in Fig. 1, the *sex*6 mutant lines and *amo*1*sex*6 double mutants contained significantly higher amylose contents than the *amo*1 mutant and wild-type lines (Fig. 2B). However, the amylose contents of *sex*6 mutant lines and *amo*1*sex*6 double mutants were not significantly different ($P$ <0.05). The amylose

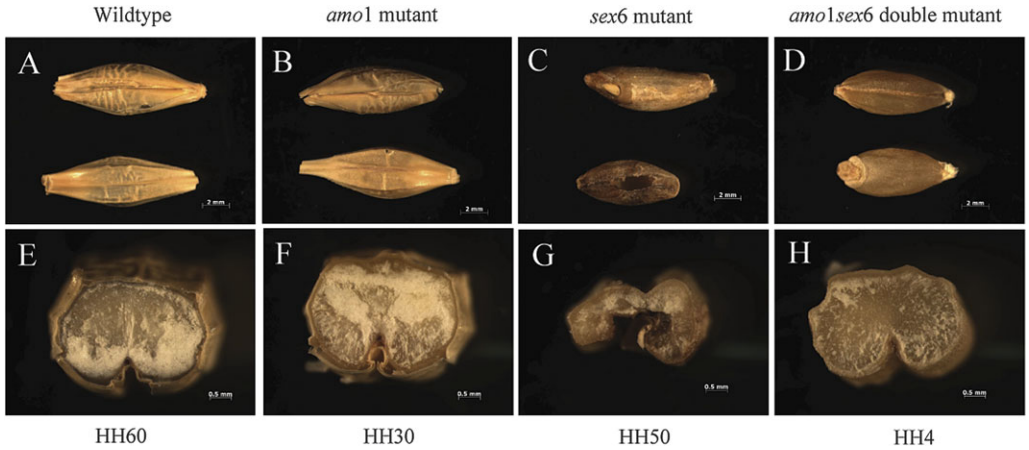


**Fig. 3.** Stereoscopic photographs of the morphology and transverse sections of grains. One line from each genotype was the wild-type line (hulled) (HH60, A and E), the *amo*1 mutant (hulled) (HH30, B and F), the *sex*6 mutant (hulless) (HH50, C and G), and the *amo*1*sex*6 double mutant (hulless) (HH4, D and I) derived from the BC3F6 population between Himalaya292 and HAG. The genotypes are labelled on the top and the lines used are given underneath. The scale bars are located in the lower right corner for each photograph.

content from *amo*1 mutant lines was also statistically significantly higher than that from the wild type ($P$ <0.05).

*Starch chain length distribution*: To examine the effects of genotype on starch chain length distribution, starch was isolated from four lines from each genotype of the BC3F6 cross population, debranched with isoamylase, and analysed by fluorophore-assisted carbohydrate electrophoresis (FACE). Figure 4 shows a difference plot in which the normalized chain length distribution (expressed as a percentage of molar molecules) for wild-type lines is subtracted from that for normalized *amo*1 mutants, *sex*6 mutants, and *amo*1*sex*6 double mutants. Lines containing the mutant *sex*6 allele (*sex*6 mutants and *amo*1*sex*6 double mutants) had more short chains (DP6–14) and fewer intermediate chains (DP15–24) compared with lines containing the wild-type *sex*6 allele (*amo*1 mutants and wild-type lines) (Fig. 4). The *amo*1 mutants had fewer short chains (DP9–14) and more intermediate chains (DP15–24) than the wild-type lines.

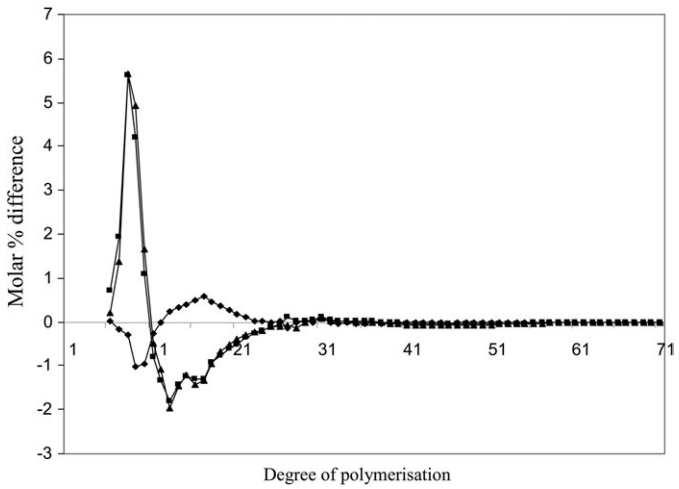


**Fig. 4.** Starch chain length distribution of the four genotypes of barley lines from the BC3F6 backcrossing population Himalaya292×HAG. Comparison of chain length distribution is expressed as a difference plot (percentage of molar molecules) between each genotype of mutant and the wild type. Three difference plots are *amo*1 mutant–wild type, diamonds; *sex*6 mutant–wild-type, squares; and *amo*1*sex*6 double mutant–wild type, triangles.

## *Other grain constituents*

Previous analysis of the *sex*6 mutation in Himalaya292 showed that the mutation was associated with pleiotropic effects on grain composition (Clarke *et al*., 2008) considered to result from redirection of carbohydrate in the grain as a result of alterations in total starch synthesis (Morell *et al*., 2003). Therefore, an analysis of the major carbohydrate, protein, and lipid fractions of the grain for each of the four genotype groups was conducted.

*Protein content*: The protein content of the four genotypes is shown in Fig. 5. The protein content of the *amo*1*sex*6 lines was significantly higher than the protein content of each of the remaining three genotypes, while the *sex*6 mutant lines contained significantly more protein than both wild-type lines and *amo*1 mutant lines ($P$ <0.05). There was no statistically significant difference ($P$ <0.05) between *amo*1 mutants and wild-type lines.

*Lipid content*: Previous studies showed that the lipid content of *sex*6 lines was elevated (Clarke *et al*., 2008). Lipid content data shown in Fig. 5 indicate that each of the four genotypes has a statistically significant difference in lipid content, with the *sex*6 genotype having the highest lipid content, followed by the *amo*1*sex*6, *amo*1, and wild-type genotypes, respectively.

β-*Glucan and pentosan content*: β-Glucan and pentosan contents of the four barley genotypes are shown in Fig. 5.

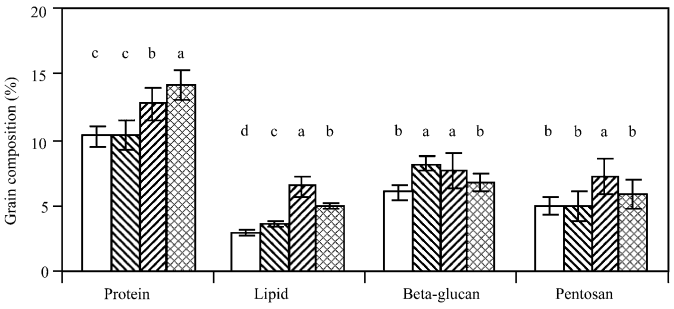


**Fig. 5.** Grain composition of the four genotypes of barley lines from the BC3F6 backcrossing population Himalaya292×HAG. The values on the *y*-axis represent the content (%) for each component as a percentage of barley grain weight. The components assayed are labelled underneath. The standard error bars are labelled on the columns. The letters (a, b, c, and d) above the columns are based on the LSD; mean values with the same letter are not statistically significantly different, and those with the different letters are statistically significantly different at $P < 0.05$. The four genotypes were the wild type (open box), *amo*1 mutant (box with downward slanting diagonal lines), *sex*6 mutant (box with upward slanting diagonal lines), and *amo*1*sex*6 double mutant (cross-hatched box).

Interestingly, these studies suggest that the *amo*1 and *sex*6 genotypes have differing impacts on cell wall polysaccharide classes. While the *amo*1 and *sex*6 genotype had higher β-glucan content than the other two genotypes, *sex*6 had the highest increase in pentosan levels. The *amo*1*sex*6 genotype had intermediate levels of both β-glucan and pentosans.

*Water-soluble carbohydrates*: Compared with the WSC composition of the grains of wild-type lines, *amo*1 mutant lines did not contain significantly different levels of total WSC, free glucose, free fructose, or maltose, but did contain significantly different levels of sucrose and fructan in their grains. However, both *sex*6 mutant lines and *amo*1*sex*6 double mutant lines contained significantly greater amounts of each of these carbohydrates than wild-type or *amo*1 lines (but not for sucrose) ($P < 0.05$) (Fig. 6). Compared with *amo*1*sex*6 double mutant lines, *sex*6 mutants contained significantly more glucose, fructose, sucrose, fructan, and total WSCs, but did not have statistically significant differences in levels of maltose compared with the *amo*1*sex*6 double mutant lines (Fig. 6).

### *Contents of starch and other grain components on a per caryopsis basis*

Expressing grain composition data on a percentage basis can be misleading when the major grain constituent, starch, differs significantly in content between genotypes, leading to apparent increases in grain constituents that do not reflect underlying synthesis rates. In order to examine the absolute levels of synthesis of the various grain components in each of the genotypes, Table 1 presents the composition data on a per caryopsis basis. This analysis confirms that, as expected, modification of the starch synthesis level is the major driver of grain weight differences between these genotypes. However, analysis of the data on this basis indicates that there have been major changes in starch synthesis among the genotypes. Each of the mutant genotypes has a decrease in amylopectin synthesis, with the *sex*6 genotype having a severe suppression of amylopectin synthesis. In contrast, the *amo*1 genotype (*amo*1 mutants and *amo*1*sex*6 double mutants) has a significant increase in amylose synthesis while the *sex*6 genotype (*sex*6 mutants) has a small decrease. The data, however, demonstrate that in the *amo*1*sex*6 genotype, the major driver of a restoration of starch content relative to the *sex*6 genotype is concomitant increases in both amylose (79% increase) and amylopectin content (61% increase).

Analysis of the levels of other grain constituents on a per caryopsis basis suggests that the protein content may be increased in the *amo*1*sex*6 genotype relative to other genotypes, lipid content is specifically increased in both the *sex6* and *amo*1*sex*6 genotypes, β-glucan and pentosan synthesis levels remain unaltered in all genotypes with the potential exception of β-glucan synthesis in the *amo*1 genotype, and WSC levels are inversely proportional to starch synthesis.

### *The* amo1 *locus negatively regulates the expression of GBSSI, SSI, and SSIIa/SBEIIa/SBEIIb proteins in the endosperm*

To investigate the mechanism underpinning the increase in amylose content on a per caryopsis basis, the level of GBSSI protein in the starch granules in barley grains was analysed from the selected lines from each of the four genotypes as GBSSI is the major enzyme involved in amylose synthesis in cereal grains. Figure 7 shows that starches from *amo*1 genotypes contain markedly higher levels of GBSSI, SSI, and SSIIa/SBEIIa/SBEIIb proteins compared with the wild type. As reported previously (Morell *et al*., 2003), starches from *sex*6 mutants contain significantly less GBSSI protein than the other three genotypes as well as no detectable SSI, or SSIIa/SBEIIa/SBEIIb proteins. Starches from *amo*1*sex*6 double mutants also contain more GBSSI protein than *sex*6 mutants, but have a similar amount of GBSSI protein to wild-type lines. However, starches from *amo*1*sex*6 double mutants do not contain any detectable traces of SSI, and SSIIa/SBEIIa/SBEIIb proteins.

### *Evidence indicating the* sslIIa *gene as the potential causal gene for the* amo1 *phenotype*

The observation that the *ssIIIa* gene encodes the only known starch synthetic enzyme closely linked with the *amo*1 locus suggests *ssIIIa* as a potential candidate gene whose mutation is causal for the *amo*1 phenotype. The presence of SSIIIa activity was examined by zymogram (enzymatic activity) from selected lines from each of the four genotypes. The results showed that there was no

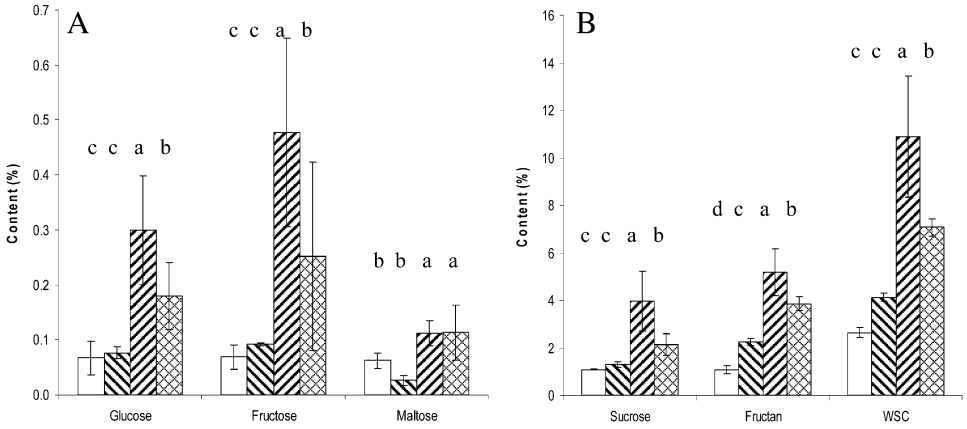


**Fig. 6.** Grain water-soluble carbohydrates of the four genotypes of barley lines from the BC3F6 backcrossing population Himalaya292×HAG. The values on the *y*-axis represent the percentage content of each component. The components assayed are labelled underneath. The standard error bars are labelled on the columns. The letters (a, b, c, and d) above the columns are based on the LSD; mean values with the same letter are not statistically significantly different, and those with different letters are statistically significantly different at $P < 0.05$. The four genotypes were the wild type (open box), *amo*1 mutant (box with downward slanting diagonal lines), *sex*6 mutant (box with upward slanting diagonal lines), and *amo*1*sex*6 double mutant (cross-hatched box). WSC, water-soluble carbohydrate.

**Table 1.** Grain constituents of barley genotypes on a per seed basis

All data are from the BC3F6 population and are expressed on a mg per seed basis. Thirteen wild-type lines, nine *amo*1 mutants, 13 *sex*6 mutants, and 13 *amo*1*sex*6 double mutants were used for the analysis of all components except WSC. Four lines from each of the four genotypes were used for WSC analysis. Three replicate samples were used for all analyses.

| | **Starch (mg)** | **Amylose (mg)** | **Amylopectin (mg)** | **Protein (mg)** |
|---|---|---|---|---|
| Wild type | 34.1±3.9 a | 10.7±1.5 b | 23.4±2.8 a | 5.4±0.7 b |
| *amo*1 | 30.2±2.5 b | 15.0±1.7 a | 15.3±1.3 b | 5.5±0.6 b |
| *sex*6 | 13.2±1.7 d | 7.7±2.1 c | 5.5±1.2 d | 4.9±0.4 c |
| *amo*1*sex*6 | 22.7±1.9 c | 13.8±1.1 a | 8.9±1.2 c | 6.5±0.5 a |
| LSD ($P$ <0.05) | 2.4 | 1.5 | 1.7 | 0.5 |
| | Lipid (mg) | β-Glucan (mg) | Pentosan (mg) | WSC (mg) |
| Wild type | 1.6±0.2 c | 3.2±0.5 b | 2.6±0.5 a | 1.4±0.2 b |
| *amo*1 | 1.9±0.2 b | 4.3±0.3 a | 2.6±0.6 a | 2.2±0.1 b |
| *sex*6 | 2.5±0.3 a | 3.0±0.4 b | 2.8±0.4 a | 3.8±1.3 a |
| *amo*1*sex*6 | 2.3±0.2 a | 3.1±0.3 b | 2.7±0.5 a | 3.3±0.4 a |
| LSD ($P$ <0.05) | 0.2 | 0.3 | 0.5 | 0.9 |

Data represent the mean of samples assayed ±SD.
Data marked with the same letter are not significantly different at $P$=0.05.

consistent change in SSIIIa activity (slow migration activity band as described by Wang *et al*., 1993; Fujita *et al*., 2007) among lines from the four genotypes (Fig 8A). Real-time reverse transcription-PCR (RT-PCR) data showed that the comparative expression for both *ssIIIa* and *ssIIIb* was similar among the four genotypes. The comparative expression for each genotype was calculated by mean values of the comparative concentration for mRNAs for *ssIIIa* and *ssIIIb* individually divided by the mean value of the comparative concentration for mRNAs for α-tubulin 2. The comparative expression levels for *ssIIIa* were between 0.15 and 0.20, while for *ssIIIb* they were between 0.018 and 0.039 (Supplementary Fig. S4 at *JXB* online). While RT-PCR suggests that both *ssIIIa* and *ssIIIb* transcripts are expressed in barley endosperm, it has not been possible to date to differentiate between SSIIIa and SSIIIb activity in zymogram analysis. However, two-dimensional affinity electrophoresis has allowed four starch synthase activities with a molecular weight consistent with SSIII to be separated (Fig. 8B). To date, it has also not been possible to assign these activities unambiguously to specific genes, and it is possible that multiple spots derive from a common gene through post-translational modifications. However, all four activities were detected from HAG (*amo*1 mutant), Glacier (a wild-type parent line for HAG), and Himalaya, providing further evidence strengthening the conclusion that the observed *amo*1 phenotype is not due to the loss of SSIIIa activity because SSIIIa activity is present in the *amo*1 mutant (Fig. 8B).

Three out of the five *amo*1*sex*6 double mutants tested showed a higher level of SSI activity compared with the *amo*1 and *sex*6 mutants. Wild-type lines had very low SSI activity (Fig. 8A). A higher level of SSI activity was also observed in the *amo*1 mutant compared with wild-type lines in two-dimensional gels (Fig. 8B). Variation in SSI and α-amylase activities was observed in the zymogram gels. The variation of α-amylase activities may be due to the different genetic backgrounds of the lines or from slight variations in the developmental stage of the endosperm material harvested.

In order to investigate whether other polymorphisms (besides the previously described SNP marker at nucleotide 6323) in the *ssIIIa* gene could underpin the *amo*1 phenotype, the cDNA from a wild-type barley Himalaya, and genomic DNA sequences from wild-type barley (Himalaya, a barley cultivar used for mutagenesis for Himalaya292), *sex*6 mutant (Himalaya292), *amo*1 mutant (HAG), and wild-type barley (Glacier, a barley cultivar used for mutagenesis for HAG) were PCR cloned and sequenced.

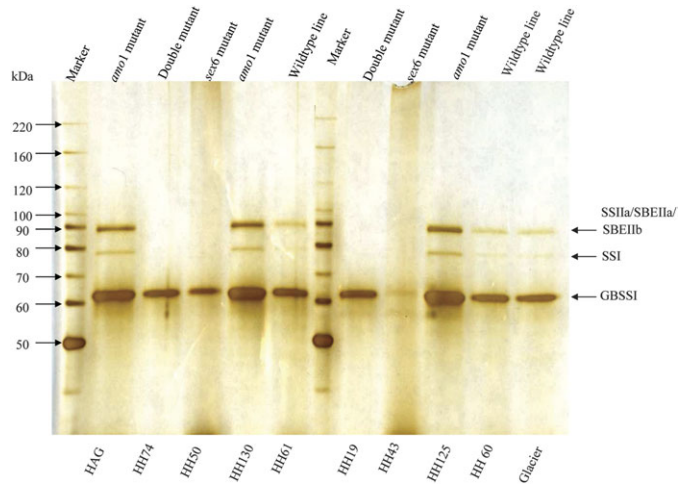


**Fig. 7.** Starch granule-bound proteins from purified starch were examined by electrophoretic analysis from the selected lines of each of the four genotypes. The genotypes are labelled on the top and the lines used are given underneath. The protein bands are identified on the right-hand side (based on Rahman *et al.*, 1995; Morell *et al.*, 2003).

The *ssIIIa* genomic DNAs from Himalaya, Himalaya292, HAG, and Glacier lines contained 9550 bp sequences with 16 exons and 15 introns (Supplementary Table S2 at *JXB* online, GenBank accession numbers: Himalaya genomic DNA, JN256944; Himalaya292 genomic DNA, JN256945, Glacier genomic DNA, JN256946; HAG genomic DNA, JN256947). The *ssIIIa* cDNA sequence from Himalaya was 5088 bp long, encoding a polypeptide with 1590 amino acid residues from nucleotide 1 to 4770 (GenBank accession number Himalaya genomic DNA, JN256948; Himalaya292 genomic DNA, JN256949, Glacier genomic DNA, JN256950; HAG genomic DNA, JN256951). Comparison between genomic DNA sequences from Himalaya292 (and Himalaya) and HAG (and Glacier) showed that there were eight SNP variations for HAG (and seven SNPs for Glacier) (Supplementary Fig. S1 and Table S2 at *JXB* online). Among them, four SNP variations for HAG (and three SNPs for Glacier) were in exons (Supplementary Fig. S2 and Table S2). Three SNPs are in exon 3 (for HAG and Glacier) and one is in exon 14 of HAG only (Supplementary Table S2). Three SNP variations at nucleotide 1084 (for HAG and Glacier), 1676 (for HAG and Glacier), and 4439 (for HAG only) of the cDNA sequences produce a change in the amino acid sequence of the protein. The first change is conservative, changing the hydrophobic amino acid methionine (Himalaya and Himalaya292) to another hydrophobic amino acid valine (HAG and Glacier) at position 362 of the protein, and the second is a conservative change from the non-polar amino acid alanine (Himalaya and Himalaya292) to a hydrophobic amino acid valine (HAG and Glacier) at position 559 of the protein, whereas the third is a non-conservative change from the hydrophobic amino acid leucine (Himalaya, Himalaya292, and Glacier) to the basic amino acid arginine (HAG) at position 1480 of the protein (Supplementaty Fig. S3).

In the five lines that showed that the linkage between the *amo*1 locus and the EBmac0501 or Bmac0090 marker was broken, all showed a linkage association between the *ssIIIa* marker and *amo*1 locus (Supplementary Table S1), indicating that the *ssIIIa* gene is the closest marker to the *amo*1 locus. An attempt was therefore made to identify lines containing a recombination between the *ssIIIa* marker used and the gene underpinning the *amo*1 phenotype, by genotyping progeny from each of the 18 heterozygous but *sex*6 mutant lines which were not used for the genotypic grouping analysis above. Grains harvested from those plants were morphologically phenotyped and all 190 lines analysed showed no recombination between the *ssIIIa* gene and the *amo*1 locus, confirming that the *ssIIIa* gene is very tightly linked to the casual gene for the *amo*1 phonotype. Further studies are required to define whether the *ssIIIa* gene is the basis of the *amo*1 mutation through down-regulation of the expression of the *ssIIIa* gene and complementation of the *amo*1 mutation by overexpression of a wild-type *ssIIIa* gene.

## Discussion

The initial objective of this study was to examine the impact on amylose content of combining recessive mutations at the *sex*6 and *amo*1 loci. Each of these mutants has increased endosperm starch amylose content, and combining these mutants might be expected to produce a higher amylose content and a concomitant decrease in starch content. However, combining the *amo*1 mutation with the *sex*6 mutation significantly restores starch synthesis in the endosperm of *sex*6 mutants through parallel increases in both amylose and amylopectin content.

The study provides further insights into the effects of the *sex*6 and *amo*1 mutations on starch synthesis. The effect of the *sex*6 mutation was to decrease amylopectin synthesis predominantly (75% reduction on a per caryopsis basis) while amylose synthesis is decreased by 28% (Morell *et al.*, 2003). Given that amylose synthesis requires the granular matrix to be present (Wattebled *et al.*, 2002), the impact of the loss of SSIIa through the *sex*6 mutation on amylose synthesis may be a secondary consequence of the major decrease in amylopectin synthesis in this mutant. It is evident that the high amylose content of the starch of s*ex*6 mutants is a consequence of the differential inhibition of amylopectin synthesis. In contrast, the data presented in Table 1 suggest that the *amo*1 mutation promotes a shift from amylopectin synthesis to amylose synthesis, resulting in the elevated amylose phenotype of the mutant. When combined with the *sex*6 mutant, the impact of *amo*1 is to increase the synthesis of both amylose and amylopectin, resulting in the significant restoration of starch synthesis and therefore grain weight in the *amo*1*sex*6 mutant, but no further increase in amylose content on a percentage basis is observed (Fig. 2). However, the amylose content on a per seed basis is significantly increased in the *amo*1*sex*6 mutant compared with the *sex*6 mutant alone (Table 1). Therefore,

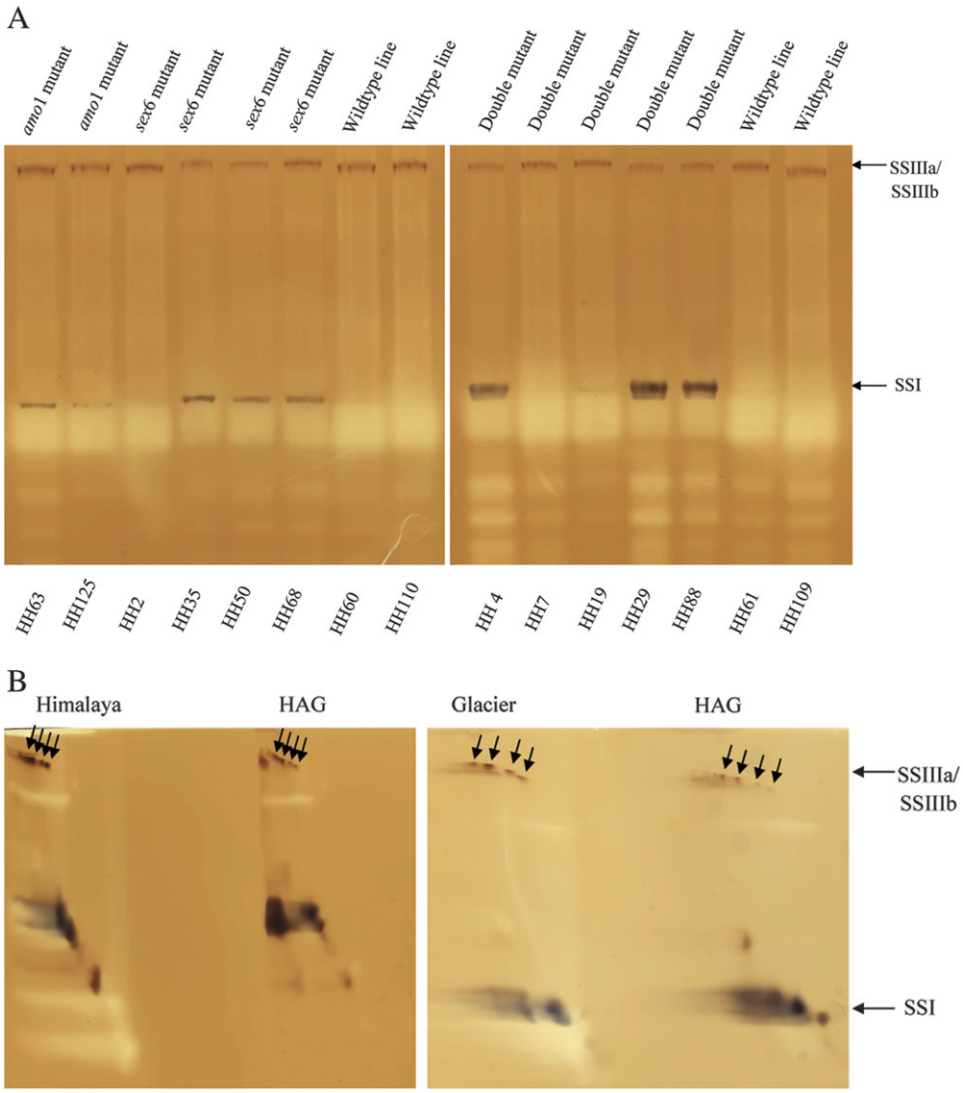


**Fig. 8.** The enzymatic activity of SSIIIa (in the presence of 1.22 mM ADP-glucose) from endosperms at 15 DPA was examined from the selected lines of each of four genotypes. (A) The enzymatic activity on the first-dimension gel. The genotypes are labelled on the top and the lines used are given underneath. (B) The enzymatic activity on the second dimension gel. The lines used are given on the top. Four arrows indicate four activities of SSIII for Himalaya, HAG (*amo*1 mutant), and Glacier. The activities of starch synthases for both A and B are on the right-hand side.

the *amo*1 mutation in both *amo*1 mutants and *amo*1*sex*6 mutants promotes the synthesis of more amylose than that from the wild-type lines. As GBSSI is essential for the biosynthesis of amylose in the endosperm (Nelson and Rines, 1962; Murata *et al*., 1965; Eriksson, 1969; Delrue *et al*., 1992; Nakamura *et al*., 1995), and also contributes to the synthesis of the long chains of amylopectin (Maddelein *et al*., 1994; Denyer *et al*., 1996), the amount of GBSSI in the starch granules in the endosperm was examined. This showed that the levels of expressed GBSSI protein in the starch granules from both *amo*1 mutants and *amo*1*sex*6 mutants were significantly increased compared with the wild-type lines and the *sex*6 mutant, respectively.

Several studies have indicated that *ssIII* is a negative regulator of starch synthesis and that a mutation in this gene can increase amylose levels. In *Arabidopsis*, studies investigating the role of the *ssIII* gene in transient starch synthesis led Zhang *et al*. (2005) to conclude that *ssIII* was a negative regulator of starch synthesis in leaves. In *Chlamydomonas*, mutations in the *ssIIIa* gene increased the amount of GBSSI protein and the level of the transcripts of GBSSI, resulting in the synthesis of more long chains in amylopectin (Ral *et al*., 2006). In rice, two *ssIII* genes are known, *ssIIIa* and *ssIIIb*. Of these genes, *ssIIIa* is expressed in the endosperm during starch synthesis whereas *ssIIIb* is expressed early in endosperm development but sharply reduces during periods of highly active starch synthesis later in grain filling (Ohdan *et al*., 2005). Mutations in the *ssIIIa* gene in rice did not change the endosperm starch content; however, there was an increase in amylose content (from 15% to 20%) suggesting that a decrease in amylopectin synthesis and a concomitant increase in amylose synthesis and the amount of GBSSI had occurred (Fujita *et al*., 2007). In maize the *dull 1* mutant which disrupts the

function of the *ssIII* gene also has an increased amylose content (Wang *et al*., 1993).

The similarities in phenotype between the *ssIIIa* and *amo*1 mutants, and the present observation that the *ssIIIa* gene maps in the same region of the genome as the *amo*1 locus in barley, suggested that *ssIIIa* is a candidate gene underpinning the *amo*1 mutation. This conclusion is strengthened by further mapping and sequencing data presented here. First, it is shown that the *ssIIIa* gene is very tightly linked to the *amo*1 locus as the *ssIIIa* gene is the closest linked marker to the *amo*1 locus and no recombinant between this gene and *amo*1 was obtained in 190 progeny lines. Secondly, sequencing of the entire *ssIIIa* gene shows that there is a difference in the amino acid sequence of the SSIIIa protein in the *amo*1 mutant compared with the reference Himalaya (or parent Glacier) gene, and this substitution (from leucine to arginine) is at conserved motif 7 within the catalytic domain of *ssIIIa* genes (Li *et al*., 2000). The activity of SSIIIa as assayed by zymogram is, however, comparable in *amo*1 and wild-type endosperm, illustrating that the total loss of SSIIIa activity does not underpin the *amo*1 phenotype.

The role of SSIIa in the elongation of amylopectin chains of DP15–24 has been previously demonstrated in barley (Morell *et al*., 2003) and extensively reviewed elsewhere for other species (Umemoto *et al*., 2004; Yamamori *et al*., 2004; Zhang *et al*., 2004; Konik-Rose *et al*., 2007). The results presented in this study suggest that the *amo*1 mutation has statistically significant effects in the chain length range DP9–14 (and DP15–24). The chain length distributions of the *sex*6 and *amo*1*sex*6 amylopectins did not show statistically significant differences across the entire distribution.

Previous studies showed that pleiotropic effects on grain composition were observed in *sex*6 mutants and that this has ramifications for understanding the impact of manipulation of starch synthesis on grain functionality and end-use, including human nutrition outcomes (Morell *et al*., 2003; Topping *et al*., 2003; Clarke *et al*., 2008). Such changes were also observed for the *amo*1 mutants and *amo*1*sex*6 double mutants. The analyses of other grain components showed that lines containing the *amo*1 mutant allele in a wild-type background had a significantly higher ($P$ <0.05) β-glucan content on a per grain basis (Table 1) than all wild-type lines. Consistent with data presented in Clarke *et al*. (2008), the *sex*6 genotypes analysed in this study had significantly higher ($P$ <0.05) lipid content and WSC content, and lower protein content on a per grain basis than the wild-type lines. With the exception of protein content (which was highest in *amo*1*sex*6 lines), the *amo*1-*sex*6 double mutants had levels of β-glucan, fibre, lipids and WSC lower than those of *sex*6 alone but inconsistent changes compared with *amo*1 mutants and the wild type (Figs 5, 6). Data presented in Table 1 suggest that only in the case of lipid and WSC are the changes in the *sex*6 and *amo*1*sex*6 genotypes associated with an increase in net synthesis per caryopsis rather than being a consequence of the dilution effect of major changes in starch content per caryopsis.

The analysis of mutations affecting starch biosynthesis in a wide range of systems (including *Chlamydomonas*, rice, maize, pea, barley, and *Arabidopsis*) has been highly informative in defining the key genes involved in the synthetic process and understanding their respective roles. However, it has frequently been noted that when combinations of mutations are generated, the effects of the mutations are not additive, suggesting that interactions between genes and gene products are important. One explanation for non-additive effects is that there is strong evidence now to demonstrate that complexes of starch biosynthetic enzymes exist and are functionally important, involving multiple isoamylases (Zeeman *et al*., 1998; Kubo *et al*., 1999) in one class of complex and branching enzymes and starch synthases in a second class of complexes (Hennen-Bierwagen *et al*., 2008; Hennen-Bierwagen *et al*., 2009; Tetlow *et al*., 2008). In barley, loss of the SSIIa protein through mutation at the *sex*6 locus results in concomitant loss of SSI, SBEIIa, and SBEIIb protein from the starch granule (Morell *et al*., 2003), possibly because of the disruption of a complex between these proteins (Hennen-Bierwagen *et al*., 2009). Furthermore, it has been noted that mutations in one starch biosynthetic gene lead to the modulation of the expression levels of other genes in the starch biosynthetic pathway (Rahman *et al*., 1995; Hylton *et al*., 1996; Yamamori *et al*., 2000; Morell *et al*., 2003; Boren *et al*., 2004; Fujita *et al*., 2007; Kosar-Hashemi *et al*., 2007). The *amo*1 mutation also increases the SSI protein level in the starch granules of the *amo*1 mutants. In maize, the *dull* 1 mutant also shows an increase in SSI activity as a result of a deficiency in SSIII (Cao *et al*., 1999). Further work needs to be carried out to resolve whether the increased content in starch granules of GBSSI (in *amo*1 and *amo*1*sex*6 genotypes), and SSI, SSIIa/SBEIIa/SBEIIb (in *amo*1 genotypes) is mediated at the gene expression, protein translation, or protein complex formation level, or are effects resulting from altered partitioning of the expressed proteins between the soluble and granular fractions.

In summary, this work demonstrated that the mutation of the *amo*1 locus up-regulates the expression of SSI, SSIIa, SBEIIa, and SBEIIb in the starch granules in barley endosperm. Genetic mapping studies indicate that the *ssIIIa* gene is tightly linked to the *amo*1 locus, and the SSIIIa protein from the *amo*1 mutant has a leucine to arginine residue substitution in a conserved domain. Zymogram analysis indicates that the *amo*1 phenotype is not a consequence of total loss of enzymatic activity, although it remains possible that the *amo*1 phenotype is underpinned by a more subtle change. It is therefore proposed that *amo*1 may be a negative regulator of other genes of starch synthesis in barley.

## Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Single nucleotide polymorphisms (SNPs) of barley *ssIIIa* genomic DNAs from HAG, Glacier, Himalaya292, and Himalaya.

Figure S2Single nucleotide polymorphisms (SNPs) of cDNA sequences of the barley *ssIIIa* gene from HAG, Glacier, Himalaya292, and Himalaya.

Figure S3. Changes in polypeptide sequences of barley SSIIIa from HAG, Glacier, Himalaya292, and Himalaya.

Figure S4. Real-time reverse transcription-PCR analysis of the expression of mRNAs for barley *ssIIIa* and *ssIIIb* of four genotypes of barley lines from the BC3F6 backcrossing population Himalaya292×HAG.

Table S1. Genotypes and phenotypes of BC3F6 lines of barley.

Table S2. Intron and exon structure of the barley *ssIIIa* gene and SNPs among barley *ssIIIa* genes from HAG, Glacier, Himalaya292, and Himalaya.

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## References

**Abel GJW, Springer F, Willmitzer L, Kossmann J.** 1996. Cloning and functional analysis of a cDNA encoding a novel 139 kDa starch synthase from potato (*Solanum tuberosum* L.). *The Plant Journal* **10,** 981–991.

**AOA Chemists.** 1990. *Official methods of analysis* Association of Official Analytical Chemists, Inc.

**Ball SG, Morell MK.** 2003. From bacterial glycogen to starch: understanding the biogenesis of the plant starch granule. *Annual Review of Plant Biology* **54,** 207–233.

**Banks W, Greenwood TC, Walker JT.** 1971. Studies on the starches of barley genotypes. A comparison of the starches from normal and high-amylose barley. *Starch/Staerke* **23,** 12–15.

**Bell BM.** 1985. A rapid method of dietary fiber estimation in wheat products. *Journal of the Science of Food and Agriculture* **36,** 815.

**Boren M, Glaring MA, Ghebremedhin H, Olsson H, Blennow A, Jansson C.** 2008. Molecular and physicochemical characterization of the high-amylose barley mutant. *Amo1. Journal of Cereal Science* **47,** 79–89.

**Borén M, Larsson H, Falk A, Jansson C.** 2004. The barley starch granule proteome—internalized granule polypeptides of the mature endosperm. *Plant Science* **166,** 617–626.

**Cao H, Imparl-Radosevich J, Guan H, Keeling PL, James MG, Myers AM.** 1999. Identification of the soluble starch synthase activities of maize endosperm. *Plant Physiology* **120,** 205–216.

**Clarke B, Liang R, Morell MK, Bird AR, Jenkins CLD, Li Z.** 2008. Gene expression in a starch synthase IIa mutant of barley: changes in the level of gene transcription and grain composition. *Functional and Integrative Genomics* **8,** 211–221.

**Clarke B, Rahman S.** 2005. A microarray analysis of wheat grain hardness. *Theoretical and Applied Genetics* **110,** 1259–1267.

**Delrue B, Fontaine T, Routier F, Decq A, Wieruszeski JM, Van den Koornhuyse N, Maddelein ML, Fournet B, Ball S.** 1992. Waxy *Chlamydomonas reinhardtii:* monocellular algal mutants defective in amylose biosynthesis and granule-bound starch synthase activity accumulate a structurally modified amylopectin. *Journal of Bacteriology* **174,** 3612–3620.

**Delvalle D, Dumez S, Wattebled F, *et al*.** 2005. Soluble starch synthase I: a major determinant for the synthesis of amylopectin in *Arabidopsis thaliana* leaves. *The Plant Journal* **43,** 398–412.

**Denyer K, Clarke B, Hylton C, Tatge H, Smith A.** 1996. The elongation of amylose and amylopectin chains in isolated starch granules. *The Plant Journal* **10,** 1135–1143.

**Devereaux J, Haeberli P, Smithies O.** 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research* **12,** 387–395.

**Eriksson G.** 1969. The waxy character. *Hereditas* **63,** 180–204.

**Fujita N, Yoshida M, Asakura N, Ohdan T, Miyao A, Hirochika H, Nakamura Y.** 2006. Function and characterization of starch synthase I using mutants in rice. *Plant Physiology* **140,** 1070–1084.

**Fujita N, Yoshida M, Kondo T, *et al*.** 2007. Characterization of SSIIIa deficient mutants of rice: the function of SSIIIa and pleiotropic effects by SSIIIa deficiency in the rice endosperm. *Plant Physiology* **144,** 2009–2023.

**Hennen-Bierwagen TA, Lin Q, Grimaud F, Planchot V, Keeling PL, James MG, Myers AM.** 2009. Proteins from multiple metabolic pathways associate with starch biosynthetic enzymes in high molecular weight complexes: a model for regulation of carbon allocation in maize amyloplasts. *Plant Physiology* **149,** 1541–1559.

**Hennen-Bierwagen TA, Liu F, Marsh RS, Kim S, Gan Q, Tetlow IJ, Emes MJ, James MG, Myers AM.** 2008. Starch biosynthetic enzymes from developing maize endosperm associate in multisubunit complexes. *Plant Physiology* **146,** 1892–1908.

**Hirose T, Terao T.** 2004. A comprehensive expression analysis of the starch synthase gene family in rice (*Oryza sativa* L.). *Planta* **220,** 9–16.

**Hylton CM, Denyer K, Keeling PL, Ming-Tang Chang M-T, Smith AM.** 1996. The effect of waxy mutations on the granule-bound starch synthases of barley and maize endosperms. *Planta* **198,** 230–237.

**James MG, Denyer K, Myers AM.** 2003. Starch synthesis in the cereal endosperm. *Current Opinion in Plant Biology* **6,** 215–222.

**Jane J-L, Chen YY, Lee LF, McPherson AE, Wong K-S, Radosavljevic M, Kasemsuwan T.** 1999. Effects of amylopectin branch chain length and amylose content on the gelatinization and pasting properties of starch. *Cereal Chemistry* **76,** 629–637.

**Konik-Rose C, Thistleton J, Chanvrier H, *et al*.** 2007. Effects of starch synthase IIa gene dosage on grain, protein and starch in endosperm of wheat. *Theoretical and Applied Genetics* **115,** 1053–1065.

**Kosar-Hashemi B, Li Z, Larroque O, Regina A, Yamamori M, Morell MK, Rahman S.** 2007. Multiple effects of the starch synthase II mutation in developing wheat endosperm. *Functional Plant Biology* **34,** 431–438.

**Kossmann J, Lloyd J.** 2000. Understanding and influencing starch biochemistry. *Critical Reviews in Plant Sciences* **19,** 171–226.

**Kubo A, Fujita N, Harada K, Matsuda T, Satoh H, Nakamura Y.** 1999. The starch-debranching enzymes isoamylase and pullulanase are both involved in amylopectin biosynthesis in rice endosperm. *Plant Physiology* **121,** 399–410.

**Li Z, Chu X, Mouille G, *et al*.** 1999. The localization, expression and role of the class II starch synthases of wheat. *Plant Physiology* **120,** 1147–1156.

**Li Z, Mouille G, Kosar-Hashemi B, Rahman S, Clarke B, Gale K, Appels R, Morell MK.** 2000. The structure and expression of the wheat starch synthase III gene: motifs in the expressed gene define the lineage of the starch synthase III gene family. *Plant Physiology* **123,** 613–624.

**Li Z, Sun F, Xu S, *et al*.** 2003. The structural organisation of the gene encoding class II starch synthase of wheat and barley and the evolution of the genes encoding starch synthases in plants. *Functional and Integrative Genomics* **3,** 76–85.

**Lunn JE, Hatch MD.** 1995. Primary partitioning and storage of photosynthate in sucrose and starch in leaves of C4 plants. *Planta* **197,** 385–391.

**Maddelein ML, Libessart N, Bellanger F, Delrue B, D'Hulst C, Van Den Koornhuyse N, Fontaine T, Wieruszeski JM, Decq A, Ball SG.** 1994. Toward an understanding of the biogenesis of the starch granule: determination of granule-bound and soluble starch synthase functions in amylopectin synthesis. *Journal of Biological Chemistry* **269,** 25150–25157.

**Morell MK, Kosar-Hashemi B, Cmiel M, Samuel MS, Chandler P, Rahman S, Buleon A, Batey IL, Li Z.** 2003. Barley *sex*6 mutants lack starch synthase IIa activity and contain a starch with novel properties. *The Plant Journal* **34,** 173–185.

**Morell MK, Li Z, Regina A, Rahman S, d'Hulst C, Ball SG.** 2006. Control of starch biosynthesis in vascular plants and algae. *Annual Plant Reviews* **22,** 258–289.

**Morrison WR, Laignelet B.** 1983. An improved colorimetric procedure determining apparent and total amylose in cereal and other starches. *Journal of Cereal Science* **1,** 9–20.

**Morrison WR, Milligan TP, Azudin MN.** 1984. A relationship between the amylose and lipid contents of starches from diploid cereals. *Journal of Cereal Science* **2,** 257–271.

**Mosse J.** 1990. Nitrogen to protein conversion factor for ten cereals and six legumes or oilgrains. A reappraisal of its definition and determination. Variation according to species and to grain protein content. *Journal of Agricultural and Food Chemistry* **38,** 18–24.

**Murata T, Sugiyama T, Akazawa T.** 1965. Enzymic mechanism of starch synthesis in glutinous rice grains. *Biochemical and Biophysical Research Communications* **18,** 371–376.

**Nakamura T, Yamamori M, Hirano H, Hidaka S, Nagamine T.** 1995. Production of waxy (amylose free) wheats. *Molecular and General Genetics* **248,** 253–259.

**Nelson OE, Rines HW.** 1962. The enzymatic deficiency in the waxy mutant of maize. *Biochemical and Biophysical Research Communications* **9,** 297–300.

**Ohdan T, Francisco PBJR, Sawada T, Hirose T, Terao T, Satoh H, Nakamura Y.** 2005. Expression profiling of genes involved in starch synthesis in sink and source organs of rice. *Journal of Experimental Botany* **56,** 3229–3244.

**O'Shea MG, Samuel MS, Konik CM, Morell MK.** 1998. Fluorophore assisted carbohydrate electrophoresis (FACE) of oligosaccharides: efficiency of labelling and high-resolution separation. *Carbohydrate Research* **307,** 1–12.

**Rahman S, Kosar-Hashemi B, Samuel MS, Hill A, Abbott DC, Skerritt JH, Preiss J, Appels R, Morell MK.** 1995. The major proteins of wheat endosperm starch granules. *Australian Journal of Plant Physiology* **22,** 793–803.

**Rahman S, Li Z, Batey I, Cochrane P, Appels R, Morell MK.** 2000. Genetic alteration of starch functionality in wheat. *Journal of Cereal Science* **31,** 91–110.

**Ral JP, Colleon C, Wattebled F, *et al*.** 2006. Circadian clock regulation of starch metabolism establishes GBSSI as a major contributor to amylopectin synthesis in *Chlamydomonas reinhardtii*.. *Plant Physiology* **142,** 305–317.

**Ramsay L, Macaulay M, degli Ivanissevich S, *et al*.** 2000. A simple sequence repeat-based linkage map of barley. *Genetics* **156,** 1997–2005.

**Roldan I, Wattebled F, Lucas MM, Delvalle D, Planchot V, Jimenez S, Perez R, Ball S, D'Hulst C, Merida A.** 2007. The phenotype of soluble starch synthase IV defective mutants of Arabidopsis thaliana suggests a novel function of elongation enzymes in the control of starch granule formation. *The Plant Journal* **49,** 492–504.

**Ruuska S, Rebetzke G, van Herwaarden A, Richards RA, Fettell N, Tabe L, Jenkins CLD.** 2006. Genotypic variation in water-soluble carbohydrate accumulation in wheat. *Functional Plant Biology* **33,** 799–809.

**Tetlow IJ, Beisel KG, Cameron S, Makhmoudova A, Liu F, Bresolin NS, Wait R, Morell MK, Emes MJ.** 2008. Analysis of protein complexes in wheat amyloplasts reveals functional interactions among starch biosynthetic enzymes. *Plant Physiology* **146,** 1878–1891.

**Tetlow IJ, Morell MK, Emes MJ.** 2004. Recent developments in understanding the regulation of starch metabolism in higher plants. *Journal of Experimental Botany* **55,** 2131–2145.

**Topping DL, Morell MK, King RA, Li Z, Bird AR, Noakes M.** 2003. Resistant starch and health—Himalaya 292, a novel barley cultivar to deliver benefits to consumers. *Starke* **55,** 539–545.

**Umemoto T, Aoki N, Lin H, Nakamura Y, Inouchi N, Sato Y, Yano M, Hirabayashi H, Maruyama S.** 2004. Natural variation in rice *starch synthase IIa* affects enzyme and starch properties. *Functional Plant Biology* **31,** 671–684.

**Wang Y-J, White P, Pollak L, Jane J.** 1993. Characterization of starch structures of 17 maize endosperm mutant genotypes with Oh43 inbred line background. *Cereal Chemistry* **70,** 171–179.

**Wattebled F, Buleon A, Bouchet B, Ral J-P, Lienard L, Delvalle D, Binderup K, Dauvillee D, Ball S, D'Hulst C.** 2002.

Granule-bound starch synthase I: a major enzyme involved in the biogenesis of B-crystallites in starch granules. *European Journal of Biochemistry* **269,** 3810–3820.

**Yamamori M, Fujita S, Hayakawa K, Matsuki J, Yasui T.** 2000. Genetic elimination of a starch granule protein, SGP-1, of wheat generates an altered starch with apparent high amylose. *Theoretical and Applied Genetics* **101,** 21–29.

**Zeeman SC, Umemoto T, Lue W-L Au-Yeung P, Martin C, Smith AM, Chen J.** 1998. A mutant of Arabidopsis lacking a chloroplastic isoamylase accumulates both starch and phytoglycogen. *The Plant Cell* **10,** 1699–1712.

**Zhang X, Colleoni C, Ratushna V, Sirghie-Colleoni M, James MG, Myers AM.** 2004. Molecular characterization demonstrates that the Zea mays gene sugary2 codes for the starch synthase isoform SSIIa. *Plant Molecular Biology* **54,** 865–879.

**Zhang X, Myers AM, James MG.** 2005. Mutations affecting starch synthase III in Arabidopsis alter leaf starch structure and increase the rate of starch synthesis. *Plant Physiology* **138,** 663–674.